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Modifications in Accessibility of Membrane Glycoproteins, Binding of Specific Ligands and Coagulation Factor V During the Activation of Platelets in Blood Emerging From Bleeding Time Wounds

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Dual color flow cytometric techniques were applied to micro-aliquots of whole blood obtained from bleeding time (BT) wounds. Modifications in platelet activation markers (P-selectin [CD62P]) and lysosomal related protein (LIMPS [CD63]), presence of membrane glycoproteins (GPIb [CD42b], GPIIb-IIIa [CD41a], GDIV [CD36], binding of von Willebrand factor (vWF), fibrinogen (Fg) and factor V [FV]) were analyzed in normal donors (n = 10) and in a severe von Willebrand patient (type 3) von Willebrand disease [vWD]). Samples of blood (20 µl) were sequentially removed from BT wound edges for up to 5 min and fixed with paraformaldehyde. Antigens were detected using the corresponding tagged monoclonal antibodies (moAbs) and quantitative results were referred to those found on platelet samples obtained from venous blood obtained from the same individuals. A progressive increase in % of platelets positive for activation dependent antigens (CD62 from 7 ± 2 to 48 ± 19% and CD63 from 9 ± 1 to 44 ± 8%; initial vs. 4 min) was observed. Accessibility of GPIIb-IIIa epitopes on platelets from BT wounds remained slightly above levels observed in venous blood platelets, despite a progressive increase in the presence of platelets positive for Fgn. Binding of MoAb to GPIV increased at late stages of BT. A moderate decrease in the binding of a moAb to GPIb was observed on platelets obtained at late stages of the BT (14 ± 9% and 20 ± 6% at 4 and 5 min, respectively). This apparent decrease in GPIb epitopes paralleled an increased presence of platelets positive for vWF (26 ± 12 and 38 ± 15%). Binding of moAb to GPIb always remained above basal levels in platelets obtained from BTs performed in the patient with type 3 vWD. FV levels on platelets coming from the BT persisted at background levels in all the individuals and at all times studied. Am. J. Hematol. 60:260–267, 1999.

Key words: bleeding time; flow cytometry; GPIb; von Willebrand factor; factor V

INTRODUCTION

Sudden disruption of vascular endothelium with exposure of the underlying subendothelium and tissue factor expression has been proposed to play a role in the mechanisms leading to hemostasis [1,2], and also in the initiation of acute thrombotic complications [3,4]. Platelet interaction with damaged vessel surfaces plays a key role in the development of acute thrombotic episodes in the arterial circulation [3]. Whereas responses of platelets to vessel elements can be analyzed through a wide variety of in vitro and ex vivo procedures, the study of alterations taking place on platelets exposed to vascular injury in vivo is difficult [5]. In fact, several clinical studies

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conducted in patients subjected to cardiopulmonary bypass or extracorporeal circulation have had limited success in finding marked expression of activation dependent antigens in circulating platelets [6–8], though this has not always been the case [9,10]. It is possible that local activation of platelets at restricted vascular regions may be transient, thus having little detectable impact on samples of platelets obtained from the general circulation.

The study of platelets emerging from bleeding time (BT) wounds offers a unique approach to the situation in which vessel subendothelium is suddenly exposed to circulating blood. Several groups have used blood collected from BT wounds to investigate the coagulation mechanism during exposure of blood to damaged vessels [1,11] and the arachidonic acid metabolism of platelets and vascular tissues [12]. More recently, this technique has been applied to investigate platelet activation. Independent studies have focused on the activation of fibrinogen receptors on platelets [8], and regulation of platelet glycoprotein Ib (GPIb) [13] or glycoprotein IV (GPIV) [14].

In the present study, we have analyzed through a wider scope modifications in the presence of platelet glycoproteins, association of their specific ligands, and some platelet-related coagulation mechanisms using a panel of specific antibodies. For this purpose, dual color flow cytometric techniques were applied to micro-aliquots of whole blood obtained from BT wounds. Modifications in platelet activation markers (P-selectin [CD62P] and lysosomal protein (LIMPS [CD63]), presence of membrane glycoproteins (GPIb [CD42b], GPIIb-IIIa [CD41a], GPIV [CD36]), binding of von Willebrand factor (vWF), fibrinogen (Fg), and cogulation factor V (FV) were analyzed in normal donors. Similar studies were conducted in a severe von Willebrand patient (type 3 von Willebrand disease [vWD]) to determine the relative role of vWF in the redistribution of major platelet glycoproteins.

MATERIALS AND METHODS

Bleeding Times and Blood Collection

The studies referred to herein were performed in 10 normal donors and a patient with type 3 vWD who was being evaluated prior to a surgical procedure and agreed to participate in the study after informed consent. All the studies were repeated twice in the type 3 patient.

A sample of blood was always obtained from the antecubital vein through a 19-gauge butterfly needle and was used as control. To avoid platelet activation, after discarding the first two ml, blood was drawn directly on 5 ml tubes containing citrate-dextrose-phosphate (citrate final concentration 119 nm/l) and 8% paraformaldehyde prepared in phosphate buffered saline (PBS) (final concentration 0.3%).

The method described by Weiss and Lages [1] was basically followed for the analysis of platelet activation in blood emerging from BT wounds. A sphygmomanometer cuff was placed on the arm and inflated up to 40 mm Hg. Two incisions were made on the volar surface of the forearm using a Simplate® II BT device (Organon Teknika Corp., Durham, NC). Blood collection was initiated after the first drop of blood appeared on the BT wounds (usually 20 to 30 sec after skin incision) [15]. Twenty μl of the blood emerging through the bleeding incisions were mixed with 300 μl of 0.3% paraformaldehyde prepared in PBS containing citrate 119 nm/L. Aliquots of whole blood were also obtained 30 sec after the first drop and every min after from BT wound edges until the bleeding stopped. Platelet counts were determined in the diluted samples of blood using phase contrast microscopy.

Antibodies

Monoclonal antibodies (moAbs) used are commercially available (Immunotech, Marseille, France) except where expressly indicated. They were purchased already tagged with fluorescein (FITC) or phycoerythrin (PE), except where expressly indicated. GPIIb-IIIa was detected with anti CD41a-PE (clone P2); GPIb with anti CD42b-FITC (clone SZ2); GPIV with anti CD36-FITC (clone FA6.152); P-selectin with anti CD62P-FITC (clone CLBThromb/6); the 53kD lysosomal membrane protein with anti CD63-FITC (clone CLBGran/12); FV with anti FV-FITC. (American Diagnostica, Greenwich, CT) [16]. Fg and vWF were detected with the corresponding polyclonal antibodies (Dako A/S, Denmark) that were conjugated previously with FITC according to standard procedures. Nonspecific membrane immunofluorescence was determined by using Ig-FITC and Ig-PE.

Flow Cytometry Studies

Immunolabeling of platelets with moAbs was performed in whole blood using dual-color analysis [8]. Briefly, after collection, 25-μl aliquots of diluted whole blood were added to polypropylene tubes preloaded with 25 μl PBS. Samples were first incubated with saturating concentrations of anti CD41a-PE in the dark, without stirring, for 15 min at room temperature, followed by the addition of FITC conjugated moAbs and another incubation for 15 min. Samples were then diluted with 1 ml PBS and analyzed.

Blood samples were analyzed with a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA) at an excitation wavelength of 488 nm. Fluorescence and scatter signals were calibrated with 2 μm Calibrite beads (Becton-Dickinson). FITC and PE fluorescence was detected with a 530/30- and 575/26-nm band pass filter, respectively, and overlap in the emission spectra of FITC and PE was corrected. Blood samples were diluted so

that the flow rate through the laser beam was less than 3,000 events per sec [17]. Platelets were differentiated from red and white cells by their specific CD41a immunofluorescence. Histograms were composed from fluorescence data obtained in the logarithmic mode; 5,000 cells were analyzed in each sample. Mean fluorescence intensities (MFI) corresponding to antibody binding (GPIb, GPIIb-IIIa, and GPIV) were expressed using the LYSYS II 1.1 conversion software (Becton-Dickinson) in an arithmetic mode on a Hewlett-Packard H-P 217 computer (Palo Alto, CA). For study of CD62P, CD63, vWF, Fg, and Fv, the results are expressed as the percentage of positive platelets for these moABs; an analytical marker was set in the green fluorescence channel to define 2% of the platelet population with the highest membrane fluorescence at the baseline level (venous sample). This marker was used as a threshold to determine the proportion of platelets exhibiting immunofluorescence above this level in all subsequent samples. All the results are expressed as mean ± standard error of the mean.

Statistical differences were analyzed by the ANOVA test for repeated measurements.

RESULTS

Number of Platelets and BTs

Bleeding in the group of normal donors stopped in 282± sec (range from 230 to 310 sec). In general, bleeding stopped in the 5 min that followed the appearance of the first drop of blood. The bleeding stopped before the fifth min in 6 of the 10 volunteers (one of them took less than 4 min) and stopped slightly beyond the fifth min in the remaining 4 volunteers. Bleeding from the wounds exceeded 15 min in the patient studied.

Number of platelets in the blood drops obtained in control donors declined along the course of the bleeding time: 180 ± 40 $\times 10^3$ plts/μl in the first drop, 170 ± 33 at 30 sec, 155 ± 35 at 1 min, 121 ± 28 at 2 min, 95 ± 45 at 3 min, 53 ± 48 at 4 min, and 23 ± 38 $\times 10^3$ plts/μl at 5 min. In the patient with type vWD, number of platelets decreased from 260 ± 8 $\times 10^3$ plts/μl in the first drop, to 254 ± 12 at 30 sec, 200 ± 10 at 1 min, 180 ± 10 at 2 min, 155 ± 15 at 3 min, 140 ± 25 at 4 min, and 120 ± 28 $\times 10^3$ plts/μl at 5 min. The progressive shortening of BTs and the reduced number of platelets at 4 and 5 min consequently influenced the number of samples (n) included for each observation times.

Platelet Activation Markers

Percentages of platelets positive for CD62P and CD63 (53 kD lysosomal protein) increased during the BT (Fig. 1A,B). A statistically significant increase in the presence of these antigens (7.1 ± 2.05% for CD62P and 9.3 ± 1.4% for CD63; $P < 0.01$ vs. venous blood) was already seen

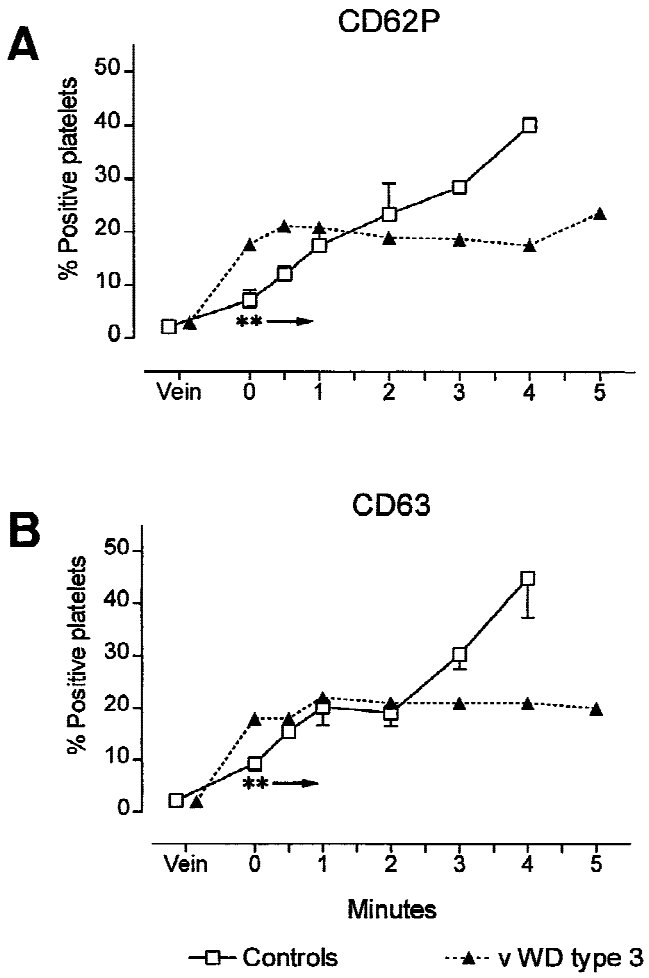

Fig. 1. Graphs show modifications in percentages of positive platelets for P-selectin (CD62P), graph A, and LIMPS (CD63), graph B, observed at different time points in blood flowing from a BT incision in control individuals and in a patient with type 3 vWD. Average numbers of platelets positive for these activation dependent antigens increased significantly in blood emerging from BT wounds vs. venous blood ($P < 0.01$ vs. venous blood). Values shown for the type 3 patient correspond to average of two different determinations. Bars represent SEM; **, $P < 0.01$ vs. venous blood, ANOVA test for repeated measurements.

in the platelets emerging with the first drop of blood that appeared from the BT incisions (usually in the 20–30 sec that followed the incision). A progressive increase in percentages of platelets positive for both intraplatelet antigens was observed during the BT procedure ($P < 0.01$ vs. venous blood at all observation times). A maximum was observed between 4 and 5 min. At those times, average percentages of positive platelets exceeded 40% for both antigens.

A similar progressive pattern expression for both CD62P and CD63 antigens was observed in those studies performed on platelets emerging from BT wounds performed in the type 3 vWD patient (Fig. 1A and B). Per-

centages of positive platelets in samples from this patient reached around 15% after the second min of bleeding and remained steady at these levels at latter observation times.

Expression of Major Platelet Glycoproteins and Association of vWF and Fg

No apparent changes were observed in the presence of GPIb in those platelets that emerged from BT wounds during the first two min. Average MFI at 0, 30 sec, 1, and 2 min remained at levels similar or slightly above those observed in platelets obtained from the venous blood samples. A progressive decrease in the availability of the GPIb antigen detected by the moAb was observed at latter observation times (Fig. 2A). At 4 min, the MFI was 86 ± 9.3% ($P < 0.05$ vs. venous blood) of that found in the venous blood and declined to 80 ± 6% in a small number of samples obtained from donors in which the BT extended beyond 5 min. As shown in Figure 2B, the study of platelet-associated vWF revealed a gradual increase in the percentage of platelets positive for this antigen during the first three min, with a more abrupt augment in blood aliquots obtained afterwards ($P < 0.05$ vs. venous blood at 3, 4, and 5 min).

An increased presence of GPIb was observed when similar studies were conducted in a patient with type 3 vWD. The fluorescence intensity was found above the levels observed in platelets obtained from venous blood in the initial sample of blood that emerged through the BT wounds and remained above 100% in further blood samples (Fig. 2A). The association of vWF to platelets remained in the background levels at all the times studied (Fig. 2B).

A slight increase in the presence of GPIIb-IIIa complexes was detected in platelets that emerged from BT wounds. The MFI showed a moderate but progressive tendency to increase in samples of blood obtained in the first 3 min ($P < 0.05$ vs. venous blood at 3 min), with an abrupt decrease in the MFI in the sample of blood obtained at the fourth min (Fig. 3A). At this moment (4 min), the MFI was still 103 ± 21% of that found in the venous blood. The study of platelet-associated Fg (Fig. 3B) revealed a slow but gradual increase in the percentage of platelets positive for this antigen during the first 3 min with a more abrupt augment in its presence when blood aliquots were obtained at later observation times ($P < 0.01$ vs. venous blood at 3, 4, and 5 min).

When similar studies were conducted in a patient with type 3 vWD, GPIIb-IIIa complexes remained basically within the levels, or slightly above, those observed in venous blood samples (Fig. 3A). The association of Fg showed a gradual tendency to increased along the BT when the study was performed in the vWD patient (Fig. 3B).

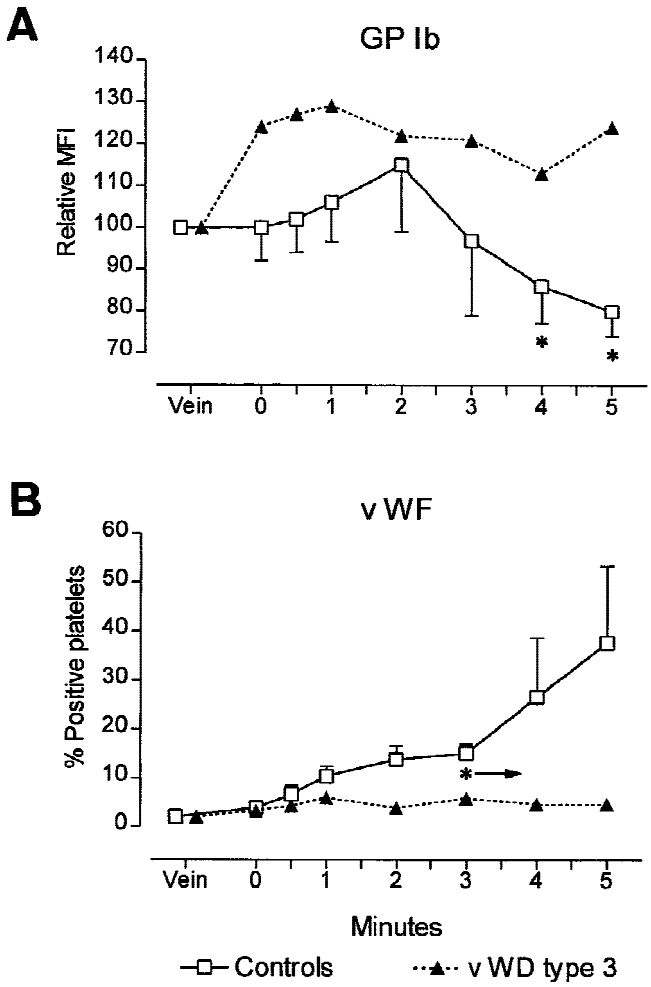

Fig. 2. Modifications in the binding of anti GPIb (CD42b), graph A, expressed as percentage of mean fluorescence intensity referred to the venous sample and percentages of positive platelets for vWF, graph B, in blood flowing from a BT incision in control individuals and in a patient with type 3 vWD (B). A moderate decrease in the binding of moAb to GPIb was observed on platelets obtained at late stages of the BTs in normal individuals. The decrease in GPIb labeling paralleled an increased presence of platelets positive for vWF. Binding of moAb to GPIb remained always above basal levels in the patient with type 3 vWD. Values shown for the type 3 patient correspond to average of two different determinations. Bars represent SEM; *, $P < 0.05$ vs. venous blood, ANOVA for repeated measurements.

Expression of GPIV

Studies in blood samples from the BTs showed that GPIV levels remained at the baseline levels in all the individuals studied (Fig. 4). Values of MFI remained steadily at values equal or above those observed in platelets from venous blood. A moderate increase in the presence of the CD36 antigen was observed in platelets from blood samples obtained at 4 min in the group of normal individuals ($P < 0.05$ vs. venous blood).

In the type 3 vWD patient, GPIV levels remained

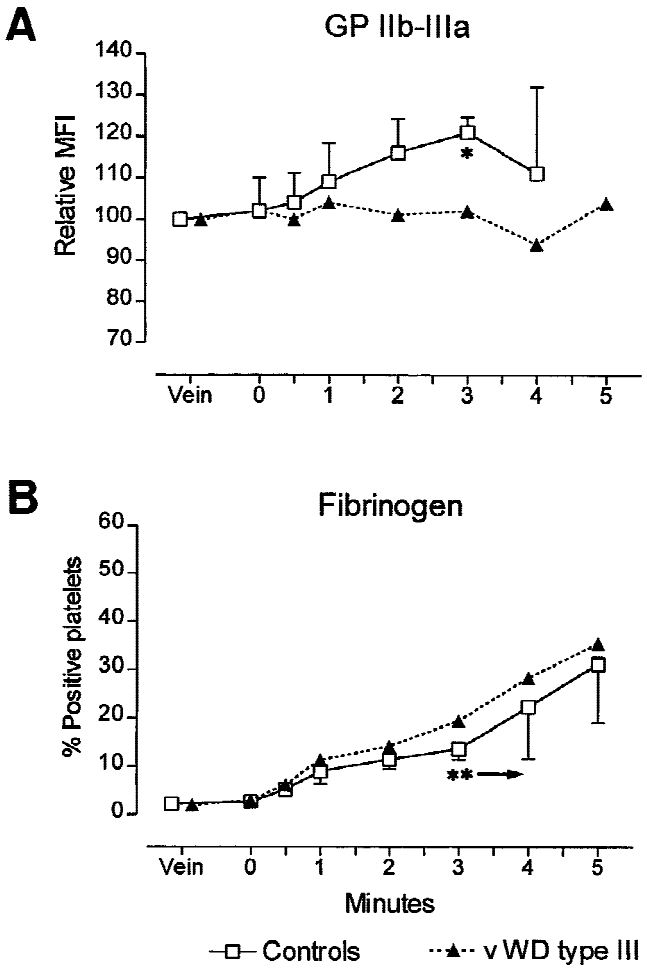

Fig. 3. Modifications in the binding of anti GPIIb-IIIa (CD41a) (A), expressed as percentage of mean fluorescence intensity referred to the venous sample and percentage of positive platelets for fibrinogen (B) in blood flowing from a BT incision in control individuals and in a patient with type 3 vWD. Accessibility of GPIIb-IIIa epitopes to the moAb used remained slightly above levels observed in venous blood platelets despite a progressive increase in the presence of platelets positive for fibrinogen. Values shown for the type 3 patient correspond to average of two different determinations. Bars represent SEM; *, $P < 0.05$, **, $P < 0.01$ vs. venous blood, ANOVA for repeated measurements.

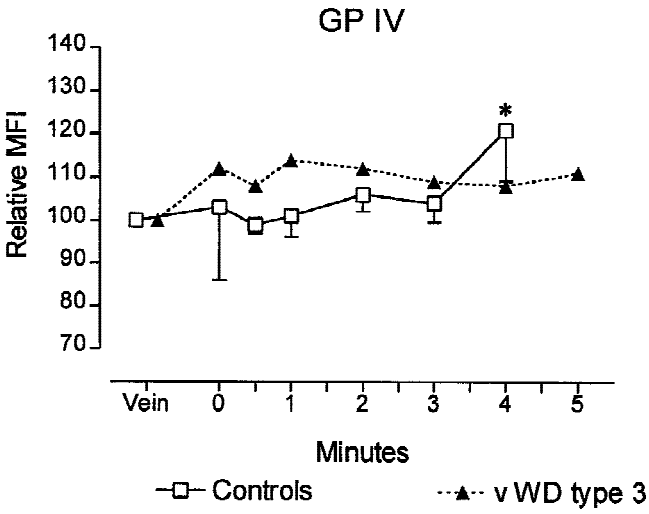

Fig. 4. Modifications in the binding of anti GPIV (CD36) expressed as percentage of mean fluorescence intensity referred to the venous sample in blood flowing from a BT incision in control individuals and in a patient with type 3 vWD. Binding of moAb to GPIV increased at late stages of BT. Values shown for the type 3 patient correspond to average of two different determinations. Bars represent SEM; *, $P < 0.05$ vs. venous blood, ANOVA for repeated measurements.

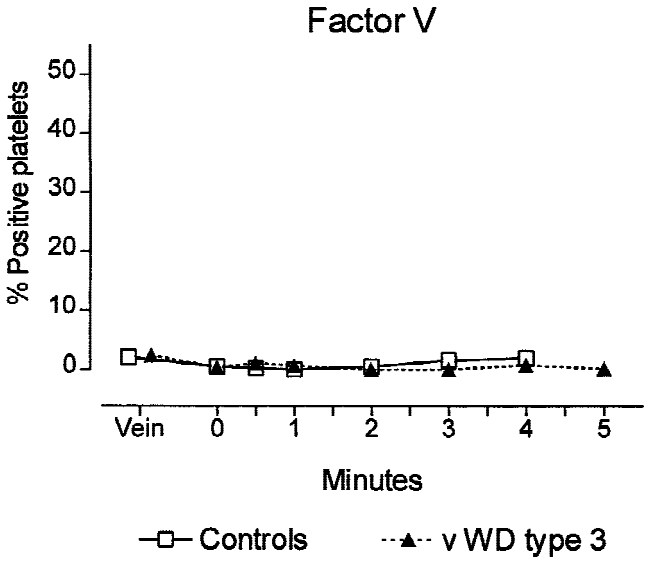

Fig. 5. Percentages of platelets positive for coagulation FV observed at different time points in blood flowing from a BT incision in control individuals and in a patient with type 3 vWD. Presence of FV persisted at background levels in all the individuals and at all times studied. Values shown for the type 3 patient correspond to average of two different determinations. Bars represent SEM.

within the levels, or slightly above, the initial levels observed in the venous samples (Fig. 4).

Expression of FV

FV levels on platelets coming from the BT persisted at background levels in all the individuals and at all times studied (Fig. 5).

DISCUSSION

Results of the present study show that platelets emerging from BT wounds are subjected to strong activation as assessed by the increased presence of platelets showing positive labeling for activation markers. Our data indicate that, under in vivo activation conditions, GPIb and GPIIb-IIIa remain in proportions that warrant their ad-

hesive and cohesive functions. Studies performed in a type 3 vWD patient GPIb suggest that this glycoprotein may in fact increase its accessibility to antibodies in platelets activated by BT wounds. Finally, our flow cytometric data suggest that exposure of platelets to the activating conditions produced at BT wounds does not result in binding of FV to the platelet surface.

When BTs are performed with template techniques with protrusion of the blade adjusted to 1 mm, the V-shaped wound incision of the skin extends 0.3 to 0.4 mm in depth and extends into the reticular layer of the dermis while transecting vessels range from 5 to 25 μm [15]. The early appearance of fibrin along the wound edges [15], the concomitant appearance of fibrinopeptide A, and the progressive increase in prothrombin fragments F_{1+2} in the bleeding time samples [18], strongly suggest that thrombin production occurs within 30 to 60 sec after the incision is made and is generated continuously at the local level along the BT wound.

P-selectin (CD62P) is an early indicator of platelet activation [19], whereas LIMPS (CD63) is expressed on the platelet surface after exposure to strong activating agents [20]. In our study, both antigens were found on the surface of platelets emerging from BT wounds. The high expression of these activation markers detected in platelets emerging from BT wounds contrasts with the usually feeble detection observed in clinical conditions caused by extracorporeal circulation of blood with extensive activation of coagulation [6,7]. However, platelet activation markers can be detected after severe vascular trauma in an experimental model [21], or when platelets are collected directly at the point where coronary angioplasty is performed [17]. One possible explanation for this apparent discrepancy is that activated platelets might have been removed from the circulation by the reticuloendothelial system. In the BT model, this possibility does not exist because platelets either attach to the wound, or become activated progressively through their exposure to thrombin and to elements of the connective tissue.

Results of in vitro and clinical studies agree on the fact that GPIIb-IIIa becomes increasingly exposed on the surface of activated platelets. Our flow cytometric date follow the same tendency, though this trend was apparently truncated when studies were performed on the fourth min. At that moment, the average MFI was still identical to that observed in samples of nonactivated platelets, and this observation was coincident with a marked increase in the presence of platelets showing positive labeling for Fg. Studies by Abrams et al. [8] using antibodies developed to specifically detect Fg bound to activated GPIIb-IIIa have shown increased association of these antibodies with activated platelets. One could assume that, despite a certain level of occupancy by their specific ligand, GPIIb-IIIa complexes remain in amounts that are equal to, or actually exceed the initial levels measured in resting platelets.

Responses of GPIb during platelet activation are controversial. Studies performed on suspensions of isolated platelets activated in vitro by thrombin have presented evidence favoring a decreased binding of moAbs to GPIb [22]. The decreased binding of moAbs to the GPIb-IX complexes, reaching 70% of the original amounts on resting platelets, has been interpreted as caused by internalization of GPIb-IX complexes into the deep portions of the open canalicular system [23]. Internalization of GPIb has been proposed as a mechanism through which thrombin might down-regulate platelet adhesion [23,24]. Interestingly, polyclonal antibodies to GPIb seem insensitive to the reductions in the binding displayed by moAbs [25].

Such impressive reductions in the binding of moAbs specific for the GPIb-related epitopes have not been found on platelets activated during cardiopulmonary bypass [26,27] or in an ex vivo thrombosis model in which circulating blood was exposed to a thrombogenic surface under conditions that allow thrombin and fibrin generation [28]. Relatively mild reductions in the presence of GPIb have been detected under some clinical conditions [10,29,30]. A single study performed on platelets activated by BT wounds [13] has reported a time-dependent decrease in the presence of platelet surface expression of GPIb. The latter study has been used frequently to link the observations on activation of isolated platelet suspensions in vitro [23,24] with those that could take place under clinical situations. Interestingly, the occurrence of proteolysis or the binding of vWF were not contemplated in the previously mentioned studies.

The presence of GPIb on platelets emerging from BT wounds increases at earlier observation times, but decreases in blood obtained after 2 min. Despite the apparent decrease in the availability of GPIb antigens, the lowest MFI in platelets emerging at 5 min was still 75% of the initial values in nonactivated platelets. Moreover, our study revealed a parallel increase in the presence of platelets showing positive labeling for vWF in blood samples obtained at the latest observation times.

Our additional studies in a severe (type 3) vWD patient, appear to explain the previous findings. The flow cytometric study performed on platelets from the type 3 patient, revealed MFI for GPIb that remained consistently above the levels noted in resting platelets. As would be expected, platelets from these patients remained always negative for vWF. Thus, the mild decrease in the binding of moAbs to GPIb observed in normal individuals may well be due to occupancy of GPIb receptors by vWF.

Usually, soluble vWF binds to GPIIb-IIIa on platelets activated by strong platelet agonists [31], or appears secreted and already bound to the GPIIb-IIIa [32]. Soluble

vWF must be exposed to exogenous modulators, such as ristocetin or botrocetin, before it can bind to the GPIb receptor [33]. The increased association of vWF to platelets in blood emerging in the latest bleeding time samples could be explained through several mechanisms. Exposure of platelets to shear stress causes binding of vWF to GPIb [34,35]. Partial proteolysis of vWF results in molecular forms that may spontaneously bind to GPIb [36]. vWF interacts with surface GPIb after contact with fibrin monomer [37]. During the BT, platelets passing through the damaged tissue are exposed to shear stress, proteolysis, and to fibrin monomers being generated at sites of vascular injury.

The overall impression of our study is that GPIb, GPIIb-IIIa, and GPIV remain on the surface of activated platelets and actually increase their expression during the activation that occurs during the exposure of platelets to the BT wound. GPIV (CD36) is a nonintegrin receptor for collagen [38] and may serve as a reference. Because the ligand remains firmly attached to the vascular wall, the receptors do not become occupied. Our flow cytometric study reveals that GPIV remains readily detectable on the platelet surface with a tendency to increase at the latest observation times, data that are in agreement with those reported previously [39]. In our opinion, an important pool of glycoproteins present in the open canalicular system becomes externalized in activated platelets. If specific ligands (vWF or Fg) are available they may bind to their receptors and make the receptors less accessible to some moAbs. Concomitant proteolysis may also affect the most sensitive epitopes of some receptors, making them unavailable to moAbs.

FV is an essential protein cofactor of the prothrombinase complex that activates prothrombin to thrombin, FV once associated to phospholipids increases the efficiency of factor Xa to generate thrombin [40]. Activation of platelets by thrombin results in the expression of FV binding sites [41]. Presumably, expression of FV on the surface of platelets that had been exposed to thrombin might be a way to extend the initial coagulation phenomenon either locally or downstream in the circulation. According to our data, platelets emerging from the BT incision, did not show substantial modification in the binding of moAb directed against FV. Recent work seems to indicate that only those platelets that have adhered to the subendothelium would be able to effectively assemble the prothrombinase complex [42]. Our studies were performed in those platelets that failed to adhere to the wound edges and this condition may have affected our results.

In summary, despite the intense activation and the possible interference of bound ligands, major glycoproteins remain on the surface of these activated platelets at levels similar or slightly greater than those found in venous blood. The quantitative reduction in the binding of the moAb to GPIb, 30% in the most evident cases, is probably related to vWF occupancy and is unlikely to justify a relevant effect on platelet adhesion.

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